EXTRACTION AND PURIFICATION OF *β-galactosidase* FROM NEW BORN SHEEP BRAIN.

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Abstract

β -galactosidase enzyme was extracted from the new born sheep brain by nine solutions, It was found that the0.2 M sodium acetate and 0.2M NaCl give a highest specific activity of crude enzyme. This enzyme was concentrated by four methods, includes: concentration by ammonium sulfate (20-60%); old acetone; ethanol (30%-60%) and by ultra filtration. It was found that the cold acetone was the better. The purification folds were about 124.78 times and yield about 77.39%. The molecular weight of this enzyme about 185.942 Kilo Dalton.

استخلاص وتنقية أنزيم *β-galactosidase* من دماغ الأغنام حديثة الولادة

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الخلاصة

تم استخلاص أنزيم β-galactosidase من دماغ الأغنام حديثة الولادة وذلك باستخدام تسعة محاليل متنوعة، وقد وجد بأن ٠.٢ مولاري من خلات الصوديوم و٠.٢ مولاري كلوريد الصوديوم هما المحلولان الأفضل لاستخلاص الأنزيم. وتم تركيز هذا الأنزيم بإتباع أربع طرائق، تتضمن التركيز باستخدام كبريتات الامونيوم (٢٠%-٦٠%) والاسيتون البارد والايثانول (٣٠%-٦٠%) والترشيح الفائق. وقد وجد أن استخدام الأسيتون البارد هي الطريقة الأفضل. وكان عدد مرات التنقية حوالي ١٢٤.٧٨ مرة والحصيلة حوالي ٧٧.٣٩%. وكان الوزن الجزيئي للأنزيم ١٨٥.٩٤٢ كيلو دالتون.

Introduction

β-galactosidase (Ec: 3.2.1.23) is the most widespread glycosidase in nature. It exists in plant and animal cells, yeast, bacteria and fungi. β-galactosidase, which is known with its common name as Lactase, is the enzyme that catalyzes the hydrolysis of β-1,4-D-galactosidic linkage between glucose and galactose of lactose molecule . [1, 2]

The extraction is the first step before purification of any enzyme and studying its characteristics .In early 1950s, the enzyme was extracted simply by water and salts solution. Then, the extraction operation has been developed by the use of different solutions; also the methods of enzyme extraction have been developed to 12 methods [3]. Since the enzymes are proteins, one of the protein extractions, such as 20% alcohol and 2-5% sodium chloride

solutions, buffer solutions of sodium phosphate or potassium can extract them. [4].

Many methods have been used to purify β-galactosiadse enzyme from different sources, like: animal, plant, or microbial sources, such as yeast, molds, and bacteria.

The enzyme was extracted from animal sources; a few attempts of purification have been made. For example, some researches tried to purify the enzyme from cow brain; they were able to purify the enzyme to the extent of homogeneity by the use of DEAE-Cellulose and then gel filtration by Sephacryl S-300 [5]. Others tried to purify β-galactosidase from cow brain as one of protective proteins [6]. While others tried to purify β-galactosidase enzyme from chicken liver by concentrating with alcohol, ionic exchange, and gel filtrate with Sephadex G-200 [7]. The researchers obtained one band when the enzyme was electrophoreses a matter that indicates the enzyme purity [8].

The objective of this study is to find a new source of β-galactosidase enzyme from animal source because this enzyme is very useful to solve the problem of lactose intolerance syndrome, also most of the studies where discussed the extraction of this enzyme from animal source is very few at all.

Materials and Methods:

-**Source of the enzyme**: The specimen (newborn sheep brain) has been prepared from a sheep in lab. This has been analyzed twice. About 10gm of new born sheep brain have been added to 100ml of de-ionized water. The mixture has been blended for 20 minutes by a magnetic mixer. Then, the mixture has been filtrated through a perforated cloth and centrifuged at a speed of 6000× g for 15 minutes. The filtrated has been kept in tightly closed tubes, and frozen at –20C^{o} until it used for estimation of the enzyme activity and protein concentration [9].

-**Determination of Moisture**, **Ash, fat and carbohydrate percentage**

has been assessed by using the method mentioned in [10].

Fat percentage has been assessed by Soxhelt method described by [11], and by petroleum ether was used to extract the fat. Carbohydrate content has been assessed on the grounds that it represents the residential materials left after subtracting the percentage of moisture, ash, protein and fat from 100 [12]. The calorific value has been assessed according to the method mentioned by [11]. pH has been assessed by using Philips 9409 device [13].

-**Extraction of the enzyme:** In order to determine the best solution for extraction of the enzyme, the following solutions have been used: extraction by distilled water [9], 0.5% sodium carbonate solution at the rate of 1:5. pH 7.2; 10% Sodium chloride solution at the rate1:4 [3]; 0.2M buffer phosphate solution pH7, 0.2 M buffer sodium acetate solution pH 5, 0.2 M buffer acetic acid solution pH 3.8, 0.2 M buffer acetate solution and 0.2 M sodium chloride pH 5, 0.06 M ascorbic acid pH 5, 0.05 M sodium acetate with 0.06 M ascorbic acid pH5.

The enzyme activity was estimated according to [14], while the protein has been estimated according to [15].

The concentration of enzyme includes; purification β-galactosidase includes

Concentration by ammonium Sulfate [16], concentration by cold acetone [3], concentration by ethyl alcohol [17] and by ultra filtration (Dutch-made Amicon-modelK52-PSi Max). XM-15 Diaflo [9].

The bio-separation methods include: ionic exchange chromatography includes: 30 gm DEAE-Cellulose (DEAE-Cellulose De-32) has been mixed with 500ml of the solution (0.25 M Sodium Hydroxide Solution, 0.25 M Buffer Sodium Chloride). The mixture has been left for 30 minutes. Then, it has been washed twice with distilled water and with stabilization solution (0.01 M Buffer Sodium Phosphate pH7) in order to reach pH7. Then, the ionic exchange has been bottled in a tube of 1.6x33cm. The column stabilization has been made with the same stabilizer until the next day at a flow speed of 0.5ml/minute [18] and gel filtration by sephadex G-100, includes: 5g of Sephadex G-100 (supplied by the Swedish Pharmacia Company) have been mixed with 250ml of distilled water. The mixture has been left in water bath at 90C^{o} for three hours in order to complete the infusion. After washing the gel with 0.1 buffer sodium acetate pH7, the mixture has been bottled in a column of 2.6 x 55cm. The column has been stabilized by the same aforementioned solution at a flow speed of 0.5ml/minute until the next day. After the ionic exchange step, the solution, which is concentrated by dilution, has been passed through the column. The same stabilizer at a flow speed of 30 ml/minute has restored the fractions, in so much as 5ml each. The light

absorbency of the restored fractions has been measured at wavelength of 280 n.m. The active fractions have been collected.

Results and Discussion

Table 1 shows the results of moisture, protein, fat carbohydrate as well as ash; 59.9%, 28.7%, 3.7%, 6.8%, and 0.9%, respectively. Also the caloric value was 173.6 kilocalorie⁄100 gm, pH 5.8 as well as the total acidity 1.3.

Table 1: Analysis of New Born Sheep Brain.

Components	Percentage (%)
Moisture	59.9
Protein	28.7
Fat	3.7
Carbohydrate	6.8
Ash	0.9
Total	100%
Caloric Value Kilo caloric⁄100gm	173.6
pH	5.8
Total acidity	1.3

Nine solutions for enzyme extraction from newborn sheep brain have been used. The results mentioned in table 2 indicate that extraction by 0.2M sodium acetate containing 0.2M NaCl pH5 is more efficiently in comparison with other extraction solutions. The specific activity and the total activity of the enzyme extracted by the above-mentioned solution were ٤6٤.٦٨٧ unit/mg and 72144 units respectively. According to the results mentioned in table 2, the high level of specific activity a long with the total activity is attributed to the increase in the ionic power which is of great effect on dismantling the ionic linkages connecting the enzyme and other components, a factor that increases the enzyme dissolution.

Table 3 shows the results derived from enzyme concentration by salting out with ammonium sulfate salt. In this case, there is a need for concentrating the enzyme solution to get rid of the materials from the crude extract as much as possible, and to get rid of water in order to increase the enzyme concentration, by concentrating; an increase of purification percentage is often achieved. The same Table shows other solutions used for enzyme concentration and explains that the ammonium sulphate solution which is widely used because it is highly dissolvable in water, does not damage the protein and its low cost in comparison with other organic dissolvent [20]. The salt concentration is gradually increased with a view to concentrating the enzyme in the crude extract, where salt balances the charges existing on protein surface, and withdraws the layer of water surrounding it, thus decreasing its dissolvable nature and increasing the protein concentration.

The enzyme purity has been determined by Poly-acrylamide Electrophoresis Under Non-denatured Conditions method [19].

Table 2: Extraction of β-galactosidase from new born sheep brain by different solutions

Extraction Solution	Volume/ ml	Activity Unit/ml	Protein Con. mg/ml	Specific activity unit/mg	Total Activity/ Unit
Distilled water	42	159	8.68	18.317	6678
Na_2Co_3 (0.5%)	47	270	7.73	34.928	12690
NaCl (10%)	46	1050	2.79	376.349	48300
0.2M Buffer Phosphate solution pH7	45	750	5.03	149.105	34200
0.2M Sodium acetate pH5	45	660	5.79	113.98	29700
0.2M Buffer Acetic Acid solution pH:3.8	47	803.4	4.48	179.33	37759
0.2M Buffer acetate solution + 0.2M NaCl pH5	48	1503	3.20	469.687	72144
0.06M Ascorbic Acid PH5	45	498.9	6.32	78.939	224505
0.05M Sodium acetate + 0.06M Ascorbic acid pH 5	46	979.3	3.59	272.785	45047

Table 3: Concentration of β-galactosidase from newborn sheep brain.

Methods	Volume (ml)	Activity Unit/ml	Protein Con. mg/ml	Specific Activity unit/mg	Total Activity Units	Purification fold	Yield (%)
Crude enzyme con. By Ammonium	48	1503	3.92	383.42	72144	1.0	100
Sulfate 20%	14	201.5	7.13	28.26	2821	0.074	3.910
60%	14	1367.3	3.00	455.76	19142.2	1.188	26.53
Crude Enzyme	47	1514	3.51	431.3	71158	1.0	100
Cold Acetone	16	3014.1	2.11	1428.48	48225.5	3.31	67.7
Crude Enzyme	48	1507	3.62	416.2	72336	1.0	100

Ethanol 30%	15	246.2	6.9	35.68	3693	0.085	5.1
60%	15	2405	1.89	1272.4	36075	3.05	49.87
Crude Enzyme Conc. by ultra filtration	46 9	1508 2297	3.70 2.09	407.5 1099	69368 20673	1.0 2.69	100 29.80

The enzyme activity was 455.76 unit/mg at 60% saturation of ammomium sulphate. This activity is slightly higher than the activity of the crude extract which is 383.41 unit/mg. The fold of purification of the enzyme concentrated by ammonium sulfate was 1.188 with a yield of 26.5%. Since this percentage and yield are less than those achieved by other solutions of concentration, so it can neglected of concentration.

Five ratios of cold acetone to enzyme extract mixtures have been used: 1:0.5, 1:1, 1:2, 1:3, 1:4 (V/V) to concentrate the enzyme. The results indicated that 1:2 rate is the best in terms of the specific activity obtained. This is why this rate has been adopted to concentrate the enzyme extract (table.3). The fold of purification achieved in this step was 3.31 times, with a yield of 67.7%. They indicated that enzyme concentration by cold acetone and alcohol is the best method in comparison with other methods.

The specific activity of the enzyme concentrated by cold ethanol was 1272.4 unit/mg when the ethanol concentration increased from 30% to 60%, while it was only 416.2 unit/mg in the crude extract. Accordingly, the fold of purification of the above concentration of ethanol was 3.05 times.

The specific activity was 1099 unit/mg with 2.69 fold of purification, with a yield of 29.8% by ultra-filtration. These results may similar to the results which concluded by [21, 7] when they used the same method to concentrate the lipoxygenase and β-galactosidase.

It can be concluded that the best concentration method is the concentration by cold acetone since it gives the highest specific activity and enzyme yield in comparison with the other four methods. When the concentration by cold acetone and DEAE Cellulose are used as a first step to purify the enzyme, it was found that the enzyme yield and purification fold 93.29 % and 20.25 respectively (table 4).

Figure 1. appears three peaks, the first one appears in the washing fraction it was totally without any activity for β-galactosidase, a matter that emphasize the binding of the enzyme with DEAE cellulose. There are two peaks in the Elution fraction; the first one contains highly β-galactosidase activity while the second peak is inactive. The highly active elution part has been collected, concentrated by freeze drying (Lyophylization) and add to the top of Sephadex-G100 column, so the specific activity was 18468.42 unit ⁄mg, purification fold 45.01 and yield 95.98%. In this step, two peaks appeared (Figure 2).

The enzyme activity was in the second peak, where purification fold increased to 45.01 times, and enzyme yield was 95.98 %.

Table 4: Purification Steps of β-galactosidase extracted from newborn sheep brain.

Step	Volume (ml)	Activity U⁄ml	Protein conc. (mg⁄ml)	Specific Activity U⁄mg	Total Activity (unit)	Purification fold	Yield 100%
Crude extract	46	1510	3.68	410.32	69460	1	100
Conc. By cold acetone	16	3001	1.27	2362.99	48016	5.759	69.13
Ion exchange chromatography DEAE-cellulose	20	3240	0.39	8307.69	64800	20.25	93.29
Gel filteration Sephadex G-100	19	3509	0.19	18468.42	66671	45.01	95.98
Gel filteration by Sephacryl S-200 (first step)	15	3471	0.12	28925	52065	70.49	74.96
Gel filteration by	15	3584	0.07	51200	53760	124.78	77.40

Sephacryl S-200 (second step)							

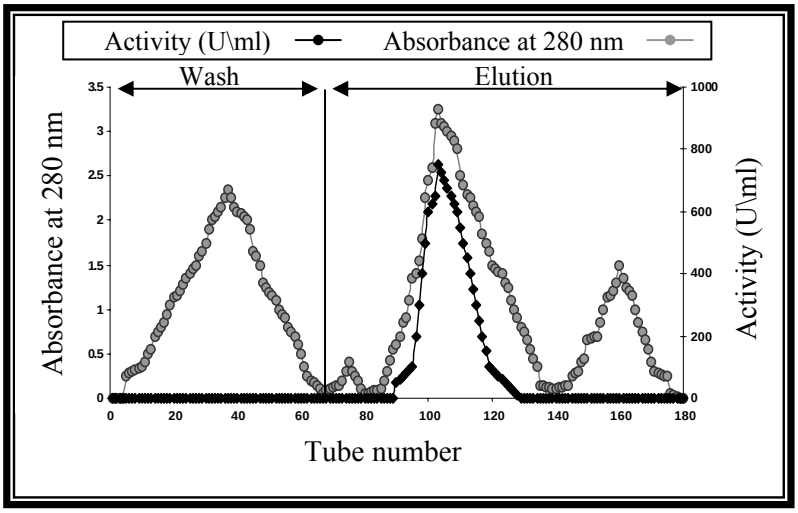

Figure 1: Ion exchange chromatography is made by using DEAE – Cellulose column (33 x 1.6 cm), and eluted with (0.01 M) Sodium phosphate buffer (pH 7) with (0 – 1 M) NaCl solution at the rate of (5 ml) for each tube.

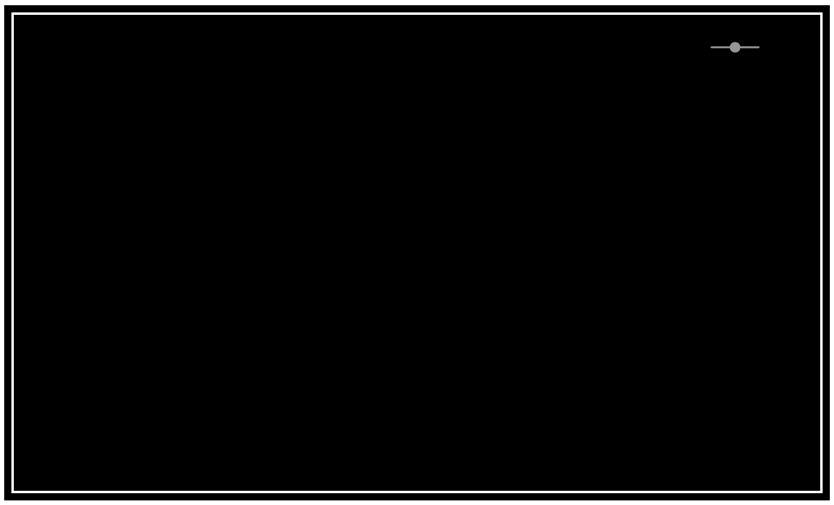

Figure 2: Gel filtration chromatography on SephadexG-100 column (2.6 x 55 Cm), eluted with 0.1M Sodium phosphate buffer (pH 7) at 5 ml for each tube.

When the enzyme was added through Spheracry Sl-200, two main peaks were appeared (figure 3), the first one not contains enzyme activity, as regards the second peak contains this activity. Purification fold is 70.49 with a yield 74.96%, The activity peak has been almost identical to the second protein peak. In order to be certain that the second protein peak is identical to the enzyme activity peak, elution parts of the latter have been passed through Sephacryl S-200 again, and under the same conditions. Only one protein peak has appeared. It has been of high enzyme activity (Figure 4). The activity peak has been completely identical to the protein activity. This initially indicates that the enzyme is absolutely pure. In this last phase, the purification fold amounts to 124.78 times, with an enzyme yield of 77.40% (Table 4).

It is noteworthy that the β-galactosidase molecular weight differs source from which it is purified. So as a result to the relationship between the elution volume, void volume and the molecular weight of standard protein

(Urease, catalase, B.S.A, Trypsin, Lysozyme and β-galactosidase) and the standard curve of β-galactosidase molecular weight (Figure 5) which determine by gel filtration using Sepharcyl S-200 column purified from newborn sheep brain, it can estimate that the β-galactosidase molecular weight is about 185.942 Kilo Dalton.

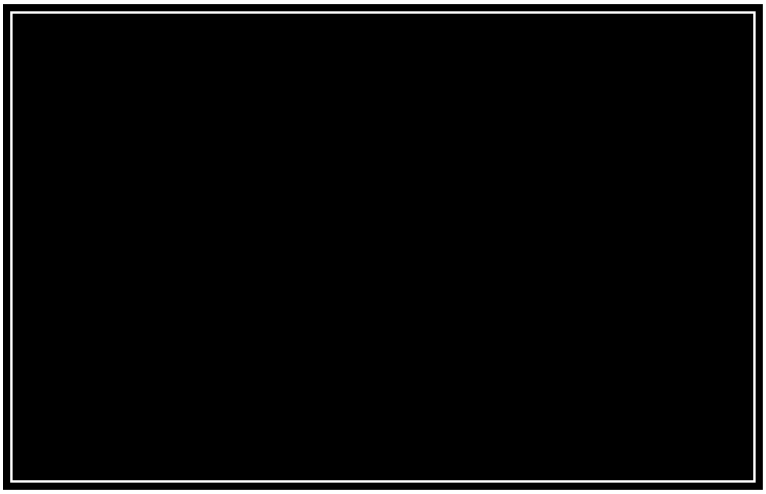

Figure 3: Gel filtration Chromatography (first step) on Sephacryl S-200 column (2.6 x 60 Cm) eluted with 0.1M Sodium phosphate buffer (pH 7) with 5 ml for each tube

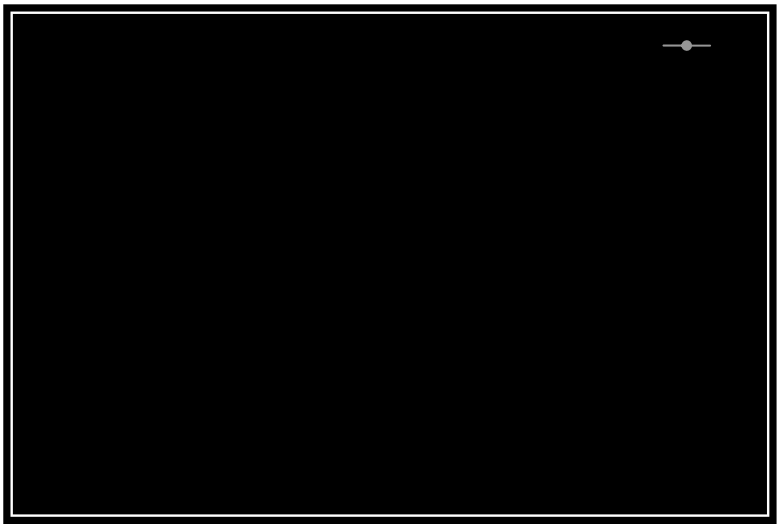

Figure 4: Gel filtration Chromatography (second step) on Sephacryl S-200 column (2.6 x 60 Cm) eluted with 0.1M Sodium phosphate buffer (pH 7) with 5 ml for each tube.

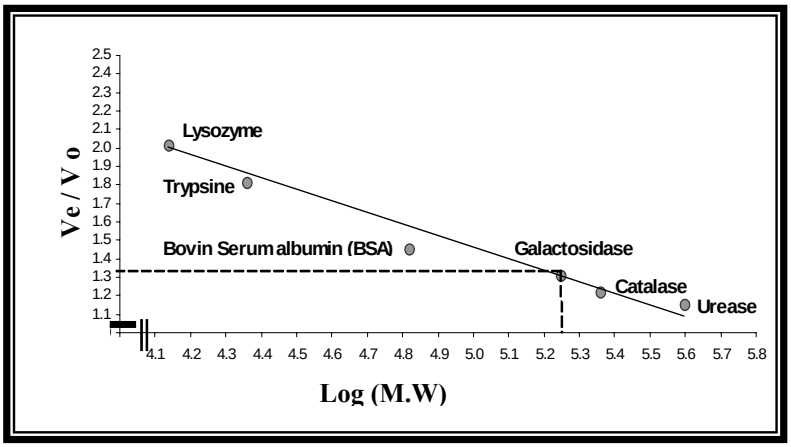

Figure 5: β-galactosidase molecular weight determination by gel filtration using S-200 column purified from new born sheep brain.

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